Sulforaphane inhibits hypoxia-induced HIF-1α and VEGF expression and migration of human colon cancer cells

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Abstract. The effects of sulforaphane (a natural product commonly found in broccoli) was investigated on hypoxia inducible factor-1α (HIF-1α) expression in HCT116 human colon cancer cells and AGS human gastric cancer cells. We found that hypoxia-induced HIF-1α protein expression in HCT116 and AGS cells, while treatment with sulforaphane markedly and concentration-dependently inhibited HIF-1α expression in both cell lines. Treatment with sulforaphane inhibited hypoxia-induced vascular endothelial growth factor (VEGF) expression in HCT116 cells. Treatment with sulforaphane modulated the effect of hypoxia on HIF-1α stability. However, degradation of HIF-1α by sulforaphane was not mediated through the 26S proteasome pathway. We also found that the inhibition of HIF-1α by sulforaphane was not mediated through AKT and extracellular signal-regulated kinase phosphorylation under hypoxic conditions. Finally, hypoxia-induced HCT116 cell migration was inhibited by sulforaphane. These data suggest that sulforaphane may inhibit human colon cancer progression and cancer cell angiogenesis by inhibiting HIF-1α and VEGF expression. Taken together, these results indicate that sulforaphane is a new and potent chemopreventive drug candidate for treating patients with human colon cancer.

Introduction

Hypoxic conditions are associated with increased tumor growth and metastasis, as well as poor survival in cancer patients (1). Hypoxia inducible factor-1α (HIF-1α) is a key protein that is expressed under hypoxic conditions and promotes vascular remodeling by vascular endothelial growth factor (VEGF). VEGF is one of the most critical factors that stimulate angiogenesis. Therefore, inhibition of HIF-1α and VEGF has demonstrated therapeutic efficacy in the treatment of several types of cancer. Expression of VEGF is regulated by hypoxia, growth factors, and oncogenes (2). HIF-1α is a heterodimeric transcription factor composed of HIF-1α and aryl hydrocarbon receptor nuclear translocator (ARNT, HIF-1β), which translocates to the nucleus and binds to hypoxia response element (HRE) binding sites. Under normoxic conditions, HIF-1α protein is efficiently degraded by the ubiquitin protein ligase Von Hippel-Lindau (VHL) and thus does not exert its effects (3). Several compounds have been tested as inhibitors of hypoxia-induced HIF-1α expression in cancer cells (4-6).

Various natural products and their analogues are currently being researched as chemopreventive agents (7-9). Sulforaphane is a potent isothiocyanate derivative found in broccoli and other vegetables, such as Brussels sprouts and cabbage, and has various health benefits, including anticancer and antioxidant properties (8,10). Many studies have revealed that sulforaphane activates phase 2 antioxidant enzymes via nuclear factor E2-related factor 2 (Nrf2) (11-14). In the last decade, many studies revealed that sulforaphane acts as a chemopreventive agent by inducing apoptosis and cell cycle arrest and inhibiting proliferation. In colon cancer cells, sulforaphane induced apoptosis and G2/M phase cell cycle arrest (15-19). In vivo studies showed that sulforaphane suppressed azoxymethane-induced colonie aberrant crypt foci (ACF) (20) and prevented polyps in Apc/Min mice (21).

Previous studies using hypoxic conditions revealed that sulforaphane inhibited expression of HIF-1α in human tongue squamous cancer cells and prostate cancer cells (7). However, the mechanisms by which sulforaphane inhibits HIF-1α in colon cancer cells under hypoxic conditions are not well understood.

In this study, we investigated the effects of sulforaphane on expression of HIF-1α and VEGF, as well as migration under hypoxic conditions, in human colon cancer cells.

Materials and methods

Chemicals. Sulforaphane was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and dissolved at a concentration of 100 mM in dimethyl sulfoxide (DMSO) as a stock solution, which was stored -20°C. The stock solution was diluted with cell

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culture medium to the desired concentration prior to use. The maximum concentration of DMSO did not exceed 0.1% (v/v), a concentration at which DMSO did not influence cell growth (data not shown). The selective proteasome inhibitor MG132 and protein synthesis inhibitor cycloheximide (CHX) were purchased from Sigma-Aldrich.

**Cell culture.** HCT116 human colon cancer cells and AGS human gastric cancer cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in RPMI-1640 medium (Hyclone, Logan, UT, USA) in a humidified atmosphere of 5% CO₂ at 37°C. The RPMI-1640 medium was supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone), 2 mM glutamine (Sigma-Aldrich), 100 U/ml penicillin (Hyclone), and 100 µg/ml streptomycin (Hyclone).

**Hypoxia experiments.** Experiments to investigate the effects of hypoxia were carried out in the hypoxia chamber of an anaerobic system (Thermo, Marietta, OH, USA). The hypoxic condition was 1% O₂ and 5% CO₂. The temperature was maintained at 37°C. The normoxic condition was 21% O₂ and 5% CO₂ (in a standard CO₂ incubator). For hypoxia experiments, HCT116 and AGS cells were grown to 50% confluence in normoxic and hypoxic chambers to allow equilibration. Immediately before each experiment, cell culture media were withdrawn from HCT116 and AGS cells and replaced with fresh media that were equilibrated to normoxic and hypoxic conditions for 24 h.

**MTT assay.** Cell survival was quantified by an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrasolium bromide; Sigma-Aldrich) assay to measure mitochondrial activity in viable cells. Cells seeded at a density of 1x10⁴ per well were allowed to adhere overnight, after which the culture media were replaced with fresh media. Cells were exposed to sulforaphane (Sigma-Aldrich) for 4 h in normoxic or hypoxic conditions. The control groups were treated with DMSO equal to the highest percentage (<0.1%) used in the experimental conditions for the MTT assay. After 4 h, the medium was replaced. MTT was freshly prepared at a concentration of 5 mg/ml in PBS and passed through a filter (pore size, 0.2 µm). An aliquot of 2 ml of MTT stock solution was added to each well and the plate was incubated at 37°C for 1 h at room temperature, after which the membranes were incubated overnight at 4°C with the primary antibodies. The membranes were washed thrice for 10 min with TBS-T buffer and incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The membranes were washed 4 times for 10 min with TBS-T buffer. Antigen-antibody complexes were detected using the enhanced chemiluminescence (ECL) detection system (GE Healthcare Biosciences, Pittsburgh, PA, USA).

**ELISA assay.** To analyze VEGF secretion, HCT116 cells were seeded in 12-well plates, cultured to 50% confluence, pretreated with sulforaphane or DMSO (control treatment) for 4 h, and switched to fresh media that were pre-conditioned in normoxic or hypoxic conditions. Cells were incubated with or without sulforaphane at the corresponding conditions for 24 h. The supernatants in the wells were collected, cleared by centrifugation, and stored at -20°C. ELISA was performed using the human VEGF Quantikine kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. Recombinant human VEGF was used for calibration. Experiments were carried out at least 3 times in triplicate.

**In vitro migration assay.** Cell migration assays were performed using 24-well modified Boyden chambers (Corning Life Science, Corning, NY, USA). A cell migration kit was used for the cell migration assay according to the manufacturer's protocol. Confluent cells were added to the inner chamber of the insert in 100 µl of serum-free medium. Medium (600 µl) with 10% FBS was added to the lower chamber. To determine the effect of sulforaphane on cell migration, 5 or 10 µM sulforaphane was added to the lower chamber (DMSO was used as a control). Cells were fixed and stained with the Diff-Quick Stain kit (Baxter, McGaw Park, IL, USA) following the procedure described by the manufacturer. The number of migrating cells was counted under a microscope (x200 magnification) and the results were expressed as the percentage of invaded cells per field for each condition.

**Statistical analysis.** Results are expressed as the mean ± SD of 3 separate experiments. Data were analyzed by Student's t-test. Means were considered significantly different at p<0.05 or p<0.01.

**Results**

Sulforaphane inhibits hypoxia-induced HIF-1α in HCT116 and AGS cells. To investigate the effects of hypoxia-induced HIF-1α, HCT116 and AGS cells were grown to 70% confluence in a standard CO₂ incubator at 37°C (normoxic conditions of 21% O₂ and 5% CO₂) and transferred to a hypoxia chamber.
Cell cultures were exposed to hypoxia (1% O₂ and 5% CO₂). Hypoxic conditions dramatically induced HIF-1α protein expression in HCT116 and AGS cells (Fig. 1A). HIF-1α protein induction was observed in cells 2 h after they were transferred to the hypoxic environment and became pronounced from the 2 h time-point through the 8 h time-point, reaching a maximum level at the 4 h and 6 h time-points; therefore, the 6 h time-point was selected for further experiments in HCT116 cells, while the 8 h time-point was selected for AGS cells.

The next study was performed to assess whether sulforaphane suppressed the observed responses to hypoxic conditions in HCT116 and AGS cells. Cells were pretreated with medium containing 12.5-100 µM sulforaphane for 1 h in normoxic conditions and transferred to a hypoxia chamber. Sulforaphane significantly inhibited HIF-1α expression in HCT116 and AGS cells (Fig. 1B). Interestingly, low concentration of sulforaphane slightly induced hypoxia-induced HIF-1α in HCT116 cells. Therefore, to investigate the effects of sulforaphane-induced cytotoxicity under normoxic and hypoxic conditions and determine whether cytotoxicity is responsible for suppression of HIF-1α accumulation, cell viability was determined by MTT assay. No significant concentration-dependent reduction of viability was observed when HCT116 and AGS cells were treated with various concentrations of sulforaphane for 6 and 8 h under normoxic and hypoxic conditions (data not shown). These data indicated that the decrease in HIF-1α abundance under normoxic and hypoxic conditions was not due to cell death.

Sulfuraphane affects the stability of HIF-1α protein in HCT116 cells. To evaluate the mechanism by which sulfuraphane inhibits HIF-1α protein expression, HCT116 cells were exposed to hypoxic conditions to induce HIF-1α protein expression. The exposure to hypoxic conditions was necessary because very little HIF-1α is detectable under normoxic conditions due to rapid protein degradation. Cyclohexamide (CHx), a protein synthesis inhibitor, is widely used in protein stability studies. HCT116 cells were incubated for 6 h under hypoxic conditions and treated with CHx for 30 min in the presence or absence of 50 µM sulforaphane. Cells were harvested at various time-points and cell lysates were subjected to western blot analysis using anti-HIF-1α antibodies. Under hypoxic conditions, the half-life of HIF-1α was not longer than 1 h when the cells were treated with CHx alone (Fig. 3A). However, the half-life of HIF-1α was ~15 min when the cells were treated with a combination of sulfuraphane and CHX (Fig. 3B). These data revealed that sulfuraphane reduced the half-life of HIF-1α protein under hypoxic conditions, indicating that sulfuraphane treatment regulates HIF-1α expression by decreasing protein stability. These results demonstrate that sulfuraphane affects hypoxia-induced HIF-1α protein stability in HCT116 cells.

Next, to examine whether sulfuraphane-induced HIF-1α protein degradation is mediated by the proteasome degradation pathway, HCT116 cells were treated with proteasome inhibitor MG132 for 30 min, followed by treatment with medium containing sulfuraphane for 30 min, after which the cells were exposed to hypoxic conditions for 6 h, washed with PBS, and

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**Figure 1. Effects of sulfuraphane on HIF-1α protein expression under hypoxic conditions in HCT116 and AGS cells. (A) Both cell lines were incubated for various time periods in a hypoxic chamber, in which cells were exposed to 1% O₂. (B) Both cell lines were exposed to 1% O₂ with various concentrations of sulfuraphane. Total cell lysates were prepared and subjected to western blot analysis. Representative results from three independent experiments are shown. β-actin was used as a loading control. N, normoxia.**
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Degradation of HIF-1α protein induced by sulforaphane under hypoxic conditions was not completely prevented by MG132 (Fig. 3C and D). These data indicate that sulforaphane did not induce HIF-1α protein degradation through the proteasome degradation pathway.

AKT and ERK signaling pathway is not involved in down-regulation of HIF-1α protein by sulforaphane under hypoxic conditions. Accumulating evidence has shown that multiple signaling pathways, particularly phosphatidylinositol 3-kinase (PI3K)/AKT and mitogen-activated protein kinase (MAPK)/ERK pathways, are involved in hypoxia-induced HIF-1α protein accumulation and downstream target gene expression (23). To determine whether sulforaphane inhibits hypoxia-mediated activation of AKT, HCT116 cells were pretreated with various concentrations of sulforaphane for 1 h under normoxic conditions, followed by incubation for 6 h under hypoxic conditions, after which the cells were washed with PBS and lysed inside the hypoxia chamber. Sulforaphane did not inhibit phosphorylation of AKT and ERK under hypoxic conditions (Fig. 4A). Interestingly, hypoxia-induced HIF-1α
was slightly increased by low concentration of sulforaphane. To determine whether sulforaphane activates AKT and ERK, cells were exposed to PI3K inhibitor LY294002 and ERK inhibitor PD98059. LY294002 and PD98059 significantly decreased the elevated levels of HIF-1α, pAKT, and pERK protein induced by hypoxic conditions (Fig. 4B). However, combination of the inhibitors with sulforaphane dramatically restored pAKT and pERK expression under hypoxic conditions (Fig. 4B). These results show that the decrease in HIF-1α expression produced by sulforaphane in HCT116 cells under hypoxic conditions is not mediated by regulation of PI3K and ERK pathways.

Sulforaphane inhibits hypoxia-induced migration. Hypoxic conditions enhance metastasis of several types of cancer cells, including colon and breast cancer cells. In vitro migration experiments were performed to determine whether sulforaphane inhibits HCT116 cell motility. HCT116 cells were treated with sulforaphane under normoxic and hypoxic conditions. As shown in Fig. 5A, there was little cell migration under normoxic conditions. However, cell migration increased under hypoxic conditions. Treatment with 5 and 10 µM sulforaphane suppressed migration activity. Sulforaphane was not cytotoxic at concentrations of 5 and 10 µM under hypoxic conditions (Fig. 5B). These results indicate that treatment with sulforaphane suppressed hypoxia-induced HCT116 cell migration.

Discussion

Phytochemicals are important cancer prevention tools. The chemopreventive mechanisms of many phytochemicals, including sulforaphane, are not well understood. Sulforaphane induces phase 2 antioxidant enzymes such as glutathione
transferases, NAD(P)H:quinone reductase, epoxide hydrolase, heme oxygenase, and UDP-glucuronosyltransferase, which play important roles in detoxification of electrophiles and protect against carcinogenesis and mutagenesis (11). During the last decade, many studies on sulforaphane as a chemopreventive agent in various cancer cell lines were released. However, the mechanism by which sulforaphane inhibits HIF-1α expression under hypoxic conditions is controversial and not well understood. In this study, we evaluated inhibition of HIF-1α and VEGF expression under hypoxic conditions by sulforaphane in human colon cancer cells.

Hypoxic conditions rapidly induced expression of HIF-1α in HCT116 human colon cancer cells and AGS human gastric cancer cells (Fig. 1A). However, sulforaphane inhibited HIF-1α expression concentration-dependently in both cell lines (Fig. 1B). Yao et al showed similar results in human tongue and prostate cancer cells (7). VEGF is a key protein downstream of HIF-1 under hypoxic conditions. Induction of VEGF expression under hypoxic conditions causes sprouting of new blood vessels from existing endothelia, which is essential for wound repair, organ regeneration, embryonic vascular system development, and a variety of pathological conditions, including tumor angiogenesis and metastasis of various solid tumors (24,25). Treatment with sulforaphane under hypoxic conditions inhibited VEGF activity in HCT116 cells (Fig. 2A). Sulforaphane also slightly suppressed HIF-1-regulated gene expressions such as VEGF, HO-1 and GLUT1 (Fig. 2B).

Degradation of HIF-1α protein under normoxic conditions is tightly regulated by ubiquitination and the 26S proteasomal degradation system. Under hypoxic conditions, ubiquitination and degradation of HIF-1α protein is suppressed, leading to stabilization and accumulation of HIF-1α protein and nuclear translocation (26). In this study, expression of HIF-1α under hypoxic conditions was inhibited by sulforaphane in HCT116 cells (Fig. 2B). Moreover, sulforaphane significantly shortened the half-life of hypoxia-induced HIF-1α protein (Fig. 3B). However, inhibition of hypoxia-induced HIF-1α protein accumulation by sulforaphane was not abolished in the presence of MG132, a potent inhibitor of the 26S proteasome (Fig. 3C). These results were similar to those reported by others previously (7). There are several possible explanations for these results. One possible explanation is that the lysosome pathway is involved in degradation of HIF-1α following sulforaphane treatment under hypoxic conditions (27). Treatment with lysosomal inhibitors, including bafilomycin A1 and chloroquine, induces HIF-1α expression and activity, while hypoxic conditions induce chaperone-mediated autophagy and lysosomal biogenesis in cancer cells (28). In addition, some studies have reported that treatment with sulforaphane strongly enhanced the proteasome pathway (29-31). Jung et al reported that hypoxia-induced HIF-1α expression was inhibited by rapamycin through interaction with von Hippel-Lindau in PC3 cells (32). Degradation of HIF-1α induced by sulforaphane under hypoxic conditions did not involve the 26S proteasome pathway in our culture system. Further studies are required to determine the mechanisms by which HIF-1α degradation induced by sulforaphane under hypoxic conditions exerts potent anti-angiogenic activity.

Accumulating evidence indicates that hypoxic conditions activate several signaling pathways, including PI3K/AKT, glycogen synthase kinase 3 beta (GSK-3β), and ERK (4,5). In this study, hypoxic conditions induced PI3K/AKT and ERK in HCT116 cells (Fig. 4A). Treatment of HCT116 cells with PI3K/AKT inhibitor LY294002 and ERK inhibitor PD98059 confirmed that both pathways are important for hypoxia-mediated HIF-1α stabilization. However, treatment with sulforaphane under hypoxic conditions induced pAKT and pERK, even in the presence of LY294002 and PD98059 (Fig. 4B). Several studies in cancer cells have shown that sulforaphane is an Nrf2 activator (8,33,34). Treatment with sulforaphane strongly and concentration-dependently induced expression of Nrf2 under hypoxic conditions in our culture system (data not shown). Treatment with sulforaphane causes autophagy in various human cancer cell lines, including colon, pancreas, and prostate cancer cells (9,35,36). Induction of PI3K and ERK pathway activity could be possible when sulforaphane causes autophagy under hypoxic conditions. However, more studies are necessary to determine the effects of sulforaphane on PI3K and ERK pathways.

Accumulating evidence in solid tumors has shown that HIF-1α overexpression, either as a result of intratumoral hypoxia or genetic alterations, activates gene transcription, and the protein products contribute to basement membrane migration and invasion (25,37,38). In this study, treatment with sulforaphane inhibited hypoxia-induced migration by HCT116 cells (Fig. 5A). The sulforaphane concentrations tested in the migration assay did not affect cell viability under hypoxic conditions (Fig. 5B). However, extensive studies are needed to identify the genes that are directly or indirectly involved in sulforaphane-regulated cancer cell migration and metastasis in response to hypoxic conditions.

In this study, we showed that sulforaphane inhibited HIF-1α protein expression in HCT116 and AGS cells under hypoxic conditions. In addition, sulforaphane treatment inhibited hypoxia-induced VEGF expression in HCT116 cells. Inhibition of HIF-1α protein expression by sulforaphane was associated with destabilization of HIF-1α protein and prevention of HIF-1α target gene activation. Sulforaphane also inhibited hypoxia-induced cell migration. These data suggest that sulforaphane may inhibit human colon cancer angiogenesis and migration by inhibiting HIF-1α and VEGF expression under hypoxic conditions. Taken together, these results indicate that sulforaphane might be a new potent chemopreventive agent against human cancer cells.

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